

AMENDMENTS TO THE SPECIFICATION

Please replace the paragraph beginning at page 1, line 21 with the following amended paragraph:

Tumor invasion and metastasis are defined as the proliferating stages of the tumor cells at whole body from the primary tumor, which finally persuade the patient into death. Cancer cells detached from the primary malignant neoplasm (mostly endothelium) penetrate the basement membrane which separates the cancer cells from the other tissue. As some penetrated cells can invade not only endothelium but also basement membrane which surrounds the blood vessels, they can migrate freely via blood vessels and finally set down on capillary vessels. If cancer cells penetrate capillary vessels again, they can form a secondary tumor. The probability of secondary tumor formation from the primary tumor followed by dissemination and invasion is under one in ten thousand (see: Erkki R., Scientific American, 257:72-77 (1996)).

Please replace the paragraph beginning at page 2, line 20 with the following amended paragraph:

The integrin receptor complex that transversely spans ~~transversely~~ the cell membrane plays a role in connecting cytoskeletal network with extracellular matrix. The core sequences, common to cell-adhesion molecules like fibrinogen, vitronectin and laminin are known to be responsible for cell adhesion, spread and integration. By the way, it is also suggested that cancer promotion and metastasis can be closely related to the role of integrin (see: Giancotti, F. G. and Rouslahti, E., Cell, 60:849-859 (1990); Hynes, R. O., Cell, 69:11-25 (1992); Nip, J. et al., J. Clin. Invest., 30 96:2096-2103 (1995)). Over-expression of fibronectin receptor $\alpha 5 \beta 1$ is known to diminish a mutated phenotype in CHO (Chinese hamster ovary) and decrease [[in]] the expression level of integrin $\alpha 5 \beta 1$ in the rodents cells which are mutated into ras (see: Plantefaben, L. C. and Hynes, R. O., 35 Cell, 56:281-290(1989)). Super-fibronectin, a fibronectin polymer, has been reported to prevent cancer promotion and metastasis (see: Pasqualini, R. et al., Nature Medicine, 2:1197-1203 (1996)).

Please replace the paragraph beginning at page 3, line 2 with the following amended paragraph:

Integrin $\alpha V \beta 3$ can be useful as a marker of most malignant tumor cells, which ~~showes~~ shows the function of integrin in the development of malignant human melanoma (see: Albelda, S. M. et

al., Cancer Res., 50:6757-6764(1990)). The expression and adhesive phenotype of integrin αV gene have a direct correlation with *in vivo* proliferation of human melanoma (see: Felding-Habermann, J. Clin. Invest., 89:2018-2022 (1992)).

Please replace the paragraph beginning at page 3, line 35 with the following amended paragraph:

Disintegrins, which are known to be potent antagonists of integrin, are small proteins derived from [[the]] snake venom (see: Niewiarowski, S. et al., Semin. Hematol., 31:289-300(1994)). Most of disintegrins contain Arg-Gly-Asp (GRD) or Lys-Gly-Asp (KGD) motifs recognized by thrombocyte fibrinogen receptor, $\alpha 2b\beta 3$. It is reported that disintegrins containing RGD sequences inhibit adhesion of integrin-mediated metastatic cells with ECM and finally block metastasis (see: Trikha, M. et al., Cancer Res., 54 (8):4993-4998(1994)). Integrin $\alpha V\beta 3$ has been identified as a marker of angiogenic vessels in chicken embryo and human cells (see: Brooks, P. C. et al., Science, 264:569-571 10(1994)). Monoclonal antibody of integrin $\alpha V\beta 3$ disrupts angiogenesis by inducing apoptosis of the newly formed vascular endothelial cells. Synthesized peptides containing RGD sequence, which is known to prevent integrin $\alpha V\beta 3$ from binding with ligands, inhibit [[a]] tumor-induced angiogenesis (see: Brooks, P. C. et al., Cell, 79:1157-1164(1994)) of CAM (chick chorioallantoic membrane). Furthermore, Angiogenin, which is known to help adhesion and proliferation of endothelial cell as a subsidiary factor, is also inhibited by synthesized RGD peptides. Recently, the snake venom-derived disintegrin, Triflavin [[is]] was reported to inhibit TNF- α stimulated angiogenesis. These results provide the possibility that disintegrins, synthesized RGD peptides and anti- $\alpha V\beta 3$ monoclonal antibodies, may be developed as anti-cancer drugs.

Please replace the paragraph beginning at page 7, line 23 with the following amended paragraph:

Figure 4a is a graph showing the effect of anti- $\alpha V\beta 3$ monoclonal antibody, GRGDSP (SEQ ID NO:7), GRGETP (SEQ ID NO:8) and recombinant Saxatilin on the binding of Vitronectin to HUVEC.

Please replace the paragraph beginning at page 7, line 26 with the following amended paragraph:

Figure 4b is a graph showing the effect of anti- α V β 3 monoclonal antibody, GRGDSP (SEQ ID NO:7), GRGETP (SEQ ID NO:8) and recombinant Saxatilin on the binding of Saxatilin to HUVEC.

Please replace the paragraph beginning at page 8, line 15 with the following amended paragraph:

The method of isolating Saxatilin from *Agkistrodon saxatilis emelianov* and the process for preparing recombinant Saxatilin are illustrated in more detail as ~~followings~~ follows.

Please replace the paragraph beginning at page 11, line 8 with the following amended paragraph:

To isolate Saxatilin from the venom of *Agkistrodon saxatilis emelianov*, 302.4 mg of crude venom from the snake was applied to a ~~Sephadex~~ SEPHADEX™ G-75 gel filtration column (1.8 x 100 cm) equilibrated with PBS buffer, and fractionated at a flow rate of 20 ml/h. The activity of Saxatilin was determined by the platelet aggregation inhibition assay: The concentrated human platelet rich plasma (PRP) obtained from 400 ml of human blood was diluted to the concentration of 300,000 platelets/ μ l. The diluted PRP (450 μ l) was mixed with 50 μ l of PBS and then incubated in the aggregometer (~~Chromo-Log~~ CHROMO-LOG™, USA) at 37 °C for 3 minutes. After collagen (2 nM) was added into the PRP solution to induce the platelet aggregation, the difference of light transmittance was measured.

Please replace the paragraph beginning at page 12, line 8 with the following amended paragraph:

The molecular weight of Saxatilin was determined as three different forms of 7,444, 7,515 and 7,647 Da by the aid of Mass spectrometer (~~Kratos Kompact Mold II, Kratos Analytical~~ KRATOS KOMPACT MOLD II™, KRATOS ANALYTICAL™, Manchester, U.K.), which is ascribed to three types of isoforms containing N-terminal sequences of 'EAGEE' and 'AGEE', in addition to 'GEE'.

Please replace the paragraph beginning at page 12, line 17 with the following amended paragraph:

For the cloning of Saxatilin cDNA, mRNA was extracted from the venom gland of *Agkistrodon saxatilis emelianov* using oligo-dT cellulose. cDNA library was constructed by using a template of mRNA obtained from the venom gland, and oligo-dT primer and reverse transcriptase. Then, 5' primer was designed by deduction from the N-terminal sequence, which is 5'-GGNGARGARTGYGAYTGYGG-3' (SEQ ID NO:3): primer 1. The 3' primer was designed by deduction from the C-terminal sequence, determined during subcloning process, which is 5'-GGCATGGAAGGGATTTCTGG-3' (SEQ ID NO:4): primer 2. The polymerase chain reaction (PCR) was performed using the 5' primer, the 3' primer and a template of the synthesized cDNA library of venom gland. The PCR products of 220 bp separated on agarose gel electrophoresis were subcloned into pGEM-T vector (~~Promega~~ PROMEGA™, USA) and used to analyze the DNA sequence encoding Saxatilin (SEQ ID NO:2). The total amino acid sequence of Saxatilin was deduced from the cloned cDNA sequence (SEQ ID NO:1).

Please replace the paragraph beginning at page 13, line 3 with the following amended paragraph:

For the comparison of the inhibitory effect of Saxatilin with those of other known peptides possessing platelet aggregation inhibitory activity, Salmosin (~~see~~: Korean Patent No. 142606, SEQ ID NO:10) and GRGDSP (SEQ ID NO:7), the platelet aggregation inhibition assay was performed as follows: 225 μ l of the human platelet rich plasma(300,000 platelets/L) was mixed with 25 μ l of the platelet aggregation inhibitor peptide solutions in PBS and then incubated in aggregometer (~~Chromo-Log~~ CHROMO-LOG™, USA) at 25 °C for 5 minutes. After ADP was added into each of the PRP solutions to induce the platelet aggregation, the turbidity was measured, respectively (~~see~~: Figure1). In Figure 1, (•) represents the degree of platelet aggregation inhibition of Saxatilin; (o), Salmosin (SEQ ID NO: 10); and (■), GRGDSP (SEQ ID NO: 7), respectively. As shown in Figure 1, IC₅₀ value of Saxatilin was 179 nM, which is comparable with 173 nM of Salmosin, and has a 1000 times more potent inhibitory effect than GRGDSP (SEQ ID NO:7) peptide.

Please replace the paragraph beginning at page 13, line 24 with the following amended paragraph:

The PCR (~~Robocycler™, Stratagene~~ ROBOCYCLER™, STRATAGENE™, USA) was performed using a plasmid containing Saxatilin cDNA, prepared from Example 4, as a template and the following two primers:

Please replace the paragraph beginning at page 13, line 35 with the following amended paragraph:

The PCR was carried out for 30 cycles of 1 min at 94 °C (denaturation), 1 min at 55 °C (annealing), and 1 min at 72 °C (polymerization), respectively. The DNA fragments of about 250 bp, PCR products, were cloned into pBluescriptKS (2.9 kb, ~~Stratagene~~ STRATAGENE™, USA) by the reaction with T4 DNA ligase to construct a recombinant plasmid. The recombinant plasmid was used for the transformation of *E.coli* XL-1Blue. The transformed *E.coli* was incubated on LB (Luria Botani) plate with 100 µg/ml of ampicillin and selected by formed white colony. The plasmid extracted from the white colony was investigated using restriction map analysis and DNA sequence analysis (~~ALF system, Amersham Pharmacia Biotech~~ ALF SYSTEM™, AMERSHAM PHARMACIA BIOTECH™, USA), and thus confirmed that the DNA fragment of 250 bp by PCR was Saxatilin cDNA. The *EcoR I/Xho I* fragment of the plasmid was cloned into the C-terminal region of the α -factor secretory signal protein of an expression vector pPIC9 (8.0 kb), to give an expression vector comprising Saxatilin encoding cDNA, pPSAX (8.3 kbp) (see: Figure 2).

Please replace the paragraph beginning at page 14, line 17 with the following amended paragraph:

The pPSAX was digested with *Sall* to give a linear DNA fragment and dissolved in TE buffer at a concentration of 0.5 µg/µl, which was then mixed with 80 µl of *Pichia pastoris* GS115 competent cell (~~Invitrogen~~ INVITROGEN™, USA) to perform transformation by electroporator (~~Bio-Rad Gene Pulser~~ BIORAD GENE PULSER™, USA) under a condition of 1.5 kV. The transformed cells were spread onto a histidine-deficient agar plate and incubated at 30 °C for 3 days. The selected colonies were inoculated to 1L of minimum glycerol media (100 mM sodium phosphate pH 6.0, yeast nitrogen base 1.34%, biotin 4x 10⁻⁵%, glycerol 1%) and incubated at 30 °C until O.D₆₀₀ unit reaches to the level of 1.0. At-the end of the incubation period, the cells were harvested by centrifugation at 3000 xg, and resuspended in the minimum methanol media (containing 100 mM sodium phosphate pH 6.0, yeast nitrogen base 1.34%, biotin 4x 10⁻⁵%,

methanol 0.5%). These cells were grown at 30 °C and induced the expression of recombinant Saxatilin. The cells were further incubated for 96 hr with the addition of 0.5% methanol at an interval of 24 hour, and it was confirmed that Saxatilin was accumulated in the media during the incubation period. The transformed cell containing Saxatilin expression vector, pPSAX, was designated as "Pichia pastoris Y/pPSAX (Pichia pastoris Y/pPSAX)", and deposited with the Korean Culture Center of Microorganisms (KCCM, Hongje-1-dong 361-221, Seodaemun-gu, Seoul, Korea), an international depository authority as accession No. KCCM-10201 on July 21, 2000.

Please replace the paragraph beginning at page 15, line 7 with the following amended paragraph:

For the purification of Saxatilin, the supernatant obtained from cell broth by the centrifugation at 5,000 xg was applied to a phenyl Sepharose column of 1.3 x 20cm (~~Bio-Rad~~ BIO RAD™, USA) equilibrated with 1.5 M ammonium sulfate solution, and eluted with 1 M ammonium sulfate solution at a flow rate of 20 ml/hour to obtain active fractions of Saxatilin. The activity of Saxatilin was determined in a similar manner as in Example 1. The active fractions were loaded into HPLC column (source 30 RPC column, 7.8 x 300mm) equilibrated in distilled water with 0.1% (v/v) TFA, and eluted by the linear gradient of 0 to 50% (v/v) acetonitrile to give pure Saxatilin with a purification yield of 107 mg/L.

Please replace the paragraph beginning at page 21, line 22 with the following amended paragraph:

As clearly illustrated and demonstrated as ~~above~~ above, the present invention provides Saxatilin, a protein derived from the venom of a Korean snake, *Agkistrodon saxatilis emelianov*, a process for preparing the same, anti-platelet aggregation agent and anti-tumor agent comprising an active ingredient of Saxatilin. The present inventors purified Saxatilin from the venom of *Agkistrodon saxatilis emelianov*, cloned cDNA encoding Saxatilin, and constructed a recombinant expression vector comprising the cDNA and a recombinant microorganism transformed with the recombinant expression vector which expresses Saxatilin, and prepared Saxatilin by culturing the recombinant microorganism.